

Split ubiquitin as a sensor of protein interactions *in vivo*

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ABSTRACT We describe an assay for *in vivo* protein interactions. Protein fusions containing ubiquitin, a 76-residue, single-domain protein, are rapidly cleaved *in vivo* by ubiquitin-specific proteases, which recognize the folded conformation of ubiquitin. When a C-terminal fragment of ubiquitin (C_{ub}) is expressed as a fusion to a reporter protein, the fusion is cleaved only if an N-terminal fragment of ubiquitin (N_{ub}) is also expressed in the same cell. This reconstitution of native ubiquitin from its fragments, detectable by the *in vivo* cleavage assay, is not observed with a mutationally altered N_{ub} . However, if C_{ub} and the altered N_{ub} are each linked to polypeptides that interact *in vivo*, the cleavage of the fusion containing C_{ub} is restored, yielding a generally applicable assay for kinetic and equilibrium aspects of *in vivo* protein interactions. This method, termed USPS (ubiquitin-based split-protein sensor), makes it possible to monitor a protein–protein interaction as a function of time, at the natural sites of this interaction in a living cell.

Multiprotein complexes mediate the bulk of biological processes (1, 2). The knowledge of these complexes is extensive for oligomeric proteins whose subunit interactions are strong enough to withstand *in vitro* conditions. However, many oligomeric assemblies, while relevant physiologically, are transient *in vivo* or unstable *in vitro*. The understanding of *in vivo* protein interactions (and especially of their temporal aspects) is still fragmentary and largely qualitative, the limitations of existing *in vivo* methods being a major reason. Assays for *in vivo* protein interactions include crosslinking of proteins with cell-penetrating reagents (2) and use of resonance energy transfer between dye-coupled proteins microinjected into cells (3). Genetic analyses of protein interactions include searches for synthetic lethal or extragenic suppressor mutations (4) which occur in genes whose products are at least functionally (and often physically) associated with a protein of interest. Another approach, the two-hybrid technique (5–8), is based on expressing one protein as a fusion to a DNA-binding domain of a transcriptional activator and expressing another protein as a fusion to a transcriptional activation domain. If the test proteins interact *in vivo*, a transcriptional activator is reconstituted, resulting in the induction of a reporter gene. This otherwise powerful method cannot address temporal aspects of a protein–protein interaction. In addition, the two-hybrid technique limits the set of detectable protein interactions to those that occur (or can be “reproduced”) in the nucleus, in proximity to the reporter gene (5–8).

We describe a ubiquitin (Ub)-based split-protein sensor (USPS; Fig. 1) that makes it possible to examine kinetic and equilibrium aspects of a protein–protein interaction at its natural sites in a living cell.

MATERIALS AND METHODS

Strains, Media, Pulse–Chase, and Immunoblotting. All experiments used the YPH500 strain of the yeast *Saccharomyces*

cerevisiae (*MAT α ura3 lys2 trp1 ade2 his3 leu2*) (11, 12) grown at 30°C to an OD_{600} of ≈ 1 in a synthetic (SD) medium (13, 14) containing 0.1 mM $CuSO_4$. Pulse–chase experiments, including the preparation of cell extracts in the presence of N-ethylmaleimide (to inhibit UBPs), immunoprecipitation with a monoclonal antibody to the “ha” epitope (14), SDS/12% PAGE, and fluorography, were carried out as described (11, 14), except that zero-time samples were withdrawn and processed 1 min after the addition of a chase medium (11). Immunoblotting with anti-ha antibody (11) was performed with the ECL detection system (Amersham).

Test Proteins. Detailed protocols are available upon request. The final constructs [verified by sequencing (15)] resided in plasmid pRS314 or pRS316 (12) and were expressed from the induced *P_{CUP1}* promoter. The *S. cerevisiae* Ub gene was amplified by PCR (15) from the previously engineered *Sal* I site immediately upstream of the Ub start codon (11) to the first cytosine of codon 37, and from codon 35 to codon 76. The primers were constructed in a way that yielded, after ligation of the two amplified fragments, a *Bam*HI site between codons 35 and 37 in the Ub open reading frame (ORF). Ligation of this ORF to a fragment encoding mouse dihydrofolate reductase with an ha epitope tag (DHFR-ha, dha) (11) yielded an ORF encoding Ub-dha (Fig. 2, construct I), which contained the sequence Met-His-Arg-Ser-Gly-Ile-Met between Gly-76 of Ub and Val-1 of DHFR. Constructs II–IV (Fig. 2) were produced by replacing the *Sal* I–*Bst*XI fragment in construct I with appropriately designed double-stranded oligonucleotides. Construct V (Fig. 2) was produced by PCR using *S. cerevisiae* genomic DNA and primers designed to amplify the region of *STE6* (16, 17) from codon 196 to codon 262. The resulting fragment was inserted into the *Bam*HI site between the Ub codons 35 and 37 in constructs I–IV, yielding constructs V–VIII. In construct IX (Fig. 2), residue 35 of Ub was preceded by a 32-residue linker all of whose residues except the N-terminal Met-Gly-Gly were specified by codons 234 to 262 of *STE6*. The z_1 - C_{ub} portion of construct XIV encoded the above *Ste6*-derived sequence preceding the C_{ub} moiety, the leucine zipper region of *S. cerevisiae* Gcn4 (residues 235–281, denoted as z_1) (18–21), the construction-generated N-terminal Met, and the sequence Gly-Glu-Ile-Ser-Thr. Constructs X–XIII (Fig. 2), derived from construct XIV and constructs V–VIII, encoded Gly-Glu-Ile-Ser-Thr-Leu-Glu C-terminally to z_1 , with Gly-Gly-Ser-Thr-Met between z_1 and N_{ub} . The z_1 motif in N_{ub}^w - z_1 and its derivatives but not in z_1 C_{ub} -dha bore a Met-250 \rightarrow Thr-250 replacement (residue numbers of Gcn4), which occurred during construction; this replacement would be expected to weaken the interaction between z_1 domains (18–21).

RESULTS

***In Vivo* Folding of Ub Containing an Insertion and/or a Single-Residue Replacement.** Ub is a 76-residue, single-

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Abbreviations: Ub, ubiquitin; USPS, Ub-based split-protein sensor; UBP, Ub-specific protease; N_{ub} , N-terminal fragment of Ub; C_{ub} , C-terminal fragment of Ub; DHFR, dihydrofolate reductase.

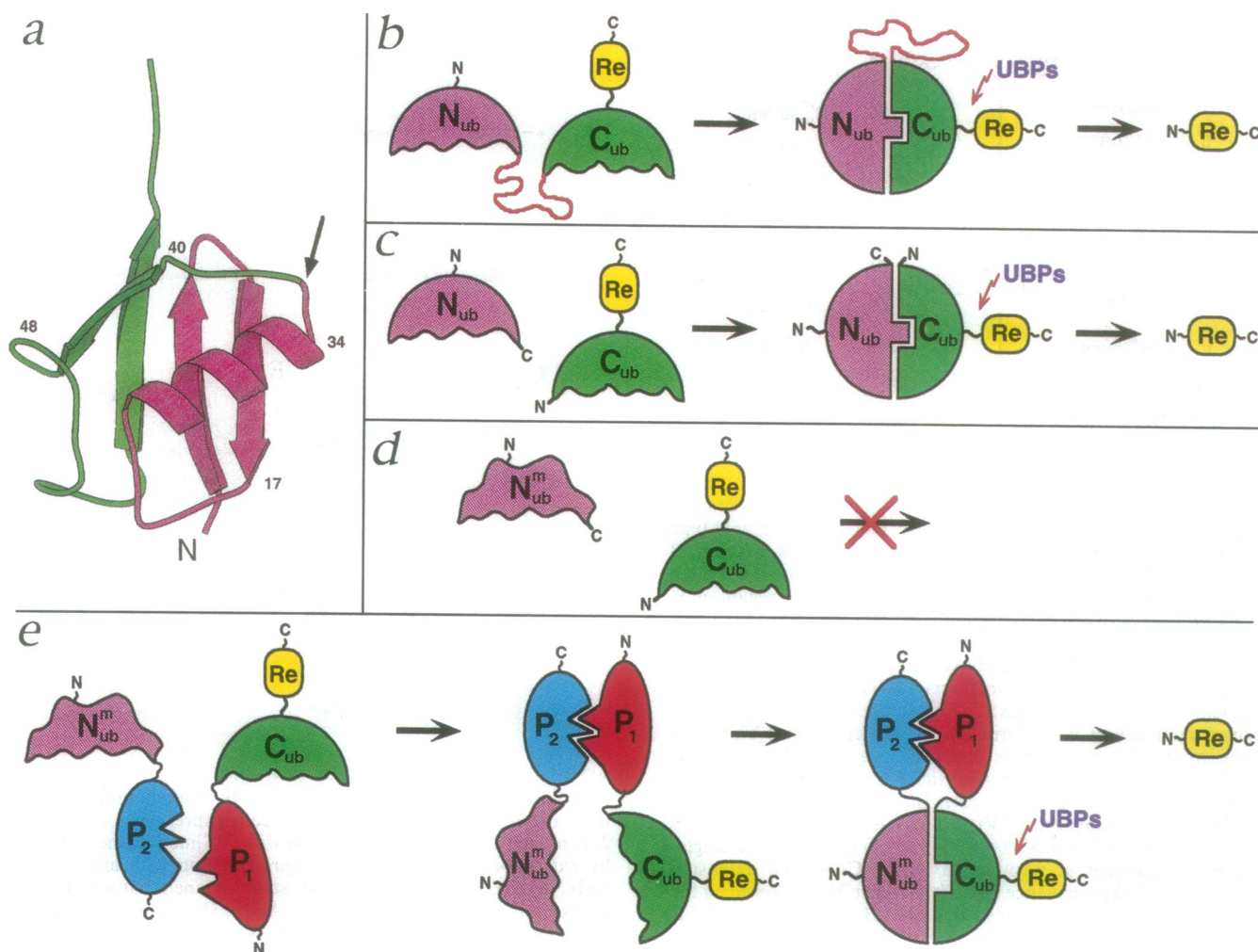


FIG. 1. Split ubiquitin as a proximity sensor *in vivo*. (a) A ribbon diagram of Ub (9, 10), with its two subdomains colored pink and green (as in b–e). Arrow denotes the site of either a 68-residue insertion or a cut between the subdomains. Some of the residue numbers are indicated. Ile-13, the site of mutations analyzed in this work, is in the second strand of the β -sheet, where it interacts with the hydrophobic face of the α -helix (9). (b) A newly formed Ub moiety bearing an insertion (wavy red line) between its N-terminal (N_{ub} ; pink) and C-terminal (C_{ub} ; green) subdomains and linked to a reporter protein (Re; yellow). The insertion did not detectably interfere with the Ub folding, which was required for the *in vivo* cleavage of the fusion by Ub-specific proteases (UBPs; red lightning arrow), yielding the free reporter. (c) When N_{ub} and C_{ub} were coexpressed as separate fragments, with C_{ub} still linked to the reporter, significant *in vivo* reconstitution of a quasi-native (recognizable by UBPs) Ub moiety was observed. (d) *In vivo* reconstitution of Ub from its separate, coexpressed fragments did not occur with a mutant N_{ub} fragment, denoted as N_{ub}^m , that bore a single-residue replacement at position 13. Conformational destabilization of N_{ub}^m relative to its wild-type counterpart N_{ub} is indicated by the altered shape of the N_{ub}^m subdomain. (e) USPS. N_{ub}^m , an altered Ub fragment that failed to reconstitute Ub in the presence of C_{ub} , did support reconstitution if the two Ub fragments were linked to polypeptides P_1 and P_2 (red and blue, respectively) that interacted *in vivo*. Reduced conformational stability of Ub that has been reconstituted with N_{ub}^m instead of N_{ub} is denoted by a gap between the Ub subdomains.

domain protein (Fig. 1a) that is present in cells either free or covalently linked to other proteins. Ub plays a role in a number of processes, primarily through routes that involve protein degradation (22–27). In eukaryotes, newly formed Ub fusions are rapidly cleaved by UBPs after the last residue of Ub at the Ub-polypeptide junction (13, 28–30). The cleavage of a Ub fusion by UBPs requires the folded conformation of Ub (11).

In the constructs of this work, Ub was joined to the N terminus of mouse DHFR, whose C terminus was extended with the ha epitope tag (14), yielding a Ub-dha test protein of 22 kDa. The Ub moiety whose Ile-3 and Ile-13 residues [which are buried in the hydrophobic core of Ub (9)] have been replaced by Gly is a poor UBP substrate (11). To make similar but less destabilizing alterations of Ub, only Ile-13 was replaced with either Val, Ala, or Gly. The resulting Ub fusions (Fig. 2, constructs II–IV) were completely cleaved *in vivo* by the end of either a 5-min or a 2-min labeling with [35 S]methionine, as was Ub-dha, bearing wild-type Ub (Fig.

3A, lanes a–d, data not shown). We then asked whether a 68-residue insertion (i68) within a loop (residues 34–40) connecting the only α -helix of Ub to a β -strand (Fig. 1a and b) and a substitution at position 13 of Ub, if present together, result in a less efficient cleavage of a fusion by UBPs. The i68 insertion was derived from the cytosolic region of the *S. cerevisiae* Ste6 protein between its transmembrane segments 4 and 5 (16, 17). This region was chosen because it was expected to be either flexible or folded in a way that positions its ends in proximity to each other. By the end of a 2-min pulse, no uncleaved Ubⁱ⁶⁸-dha, and at most traces of Ub^{V13,i68}-dha, could be detected (Fig. 2, constructs V and VI; 3B, lanes a and b). However, the cleavage of Ub^{A13,i68}-dha and especially Ub^{G13,i68}-dha was much slower, in that significant amounts of the uncleaved fusions were observed by the end of a 2-min pulse (Fig. 2, constructs VII and VIII; Fig. 3B, lanes c and d).

The i68 insertion places the two “halves” of a nascent Ub farther apart (Fig. 1a and b) and therefore is expected to

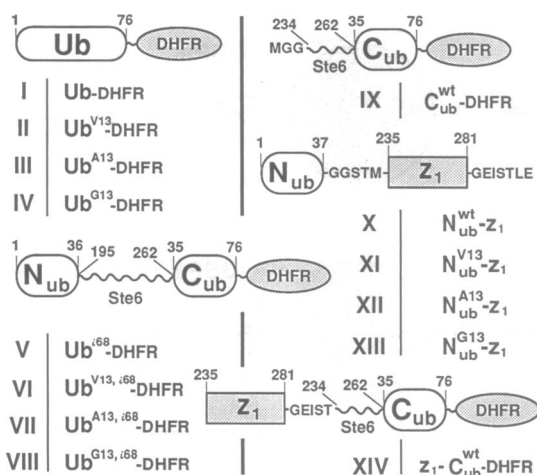


FIG. 2. Fusion constructs. Fusions used in this work contained some of the following elements: (i) a Ub moiety, either wild-type (construct I) or bearing single-residue replacements at position 13 (constructs II–IV). (ii) A Ub moiety containing the 68-residue insertion (denoted as Ste6) derived from the cytosolic region of *S. cerevisiae* Ste6 between its transmembrane segments 4 and 5 (16, 17). The insertion was positioned after residue 36 of Ub (construct V). (iii) A Ub moiety bearing both the above insertion and a single-residue replacement at position 13 (constructs VI–VIII). (iv) A C-terminal fragment of Ub (C_{ub} , residues 35–76) bearing the 32-residue, Ste6-derived sequence at its N terminus (construct IX). (v) The same fusion whose N terminus was extended, via the linker sequence Gly-Glu-Ile-Ser-Thr, with the 47-residue homodimerization motif ("leucine zipper", or z_1) of *S. cerevisiae* Gcn4 (18–21) (residues 235–281 of Gcn4) (construct XIV). (vi) An N-terminal fragment of Ub (N_{ub} , residues 1–37) bearing the wild-type Ub sequence or a single-residue replacement at position 13 and a C-terminal extension containing the linker sequence Gly-Gly-Ser-Thr-Met followed by the z_1 leucine zipper of Gcn4 (constructs X–XIII). (vii) Mouse DHFR bearing a C-terminal ha epitope (14) (denoted as DHFR in this diagram and as dha in the text and in Figs. 3 and 4).

retard the folding of Ub; this effect could be detected by the 2-min pulse-cleavage assay if another destabilizing Ub alteration such as Ile-13 → Gly-13 (which by itself is insufficient to cause a detectable retardation of Ub folding) was present as well (Fig. 3 A and B). These results can be interpreted within the diffusion-collision model of protein folding (2, 31, 32), in which marginally stable units of isolated secondary structure form early and then coalesce into the native conformation. Indeed, in native Ub, its first 34 residues are folded into an α -helix interacting with a double-stranded antiparallel β -sheet (Fig. 1a) (9). Thus, the i68 insertion retards Ub folding primarily through a reduction in the frequency of collisions between the N-terminal and C-terminal subdomains of Ub, whereas the effect of substitutions at position 13 of Ub is a decreased conformational stability of its N-terminal subdomain (Fig. 1a). This *in vivo* evidence for Ub subdomains (see also below) is in agreement with the results of circular dichroism and NMR analyses of Ub and its fragments in a methanol/water solvent at low pH (33, 34).

In Vivo Reconstitution of Ub from Its Fragments. The relative insensitivity of Ub folding to a large insertion within the 34–40 loop (Fig. 1 a and b) suggested that separate, coexpressed fragments of Ub (produced by a cut within the 34–40 loop) may be able to reconstitute native Ub. In a test of this conjecture, a C-terminal fragment of wild-type Ub (residues 35–76, denoted as C_{ub}) was expressed as a fusion to the dha reporter (C_{ub} -dha), while an N-terminal fragment of wild-type Ub (residues 1–37, denoted as N_{ub}) was expressed as a fusion to the "leucine zipper" homodimerization domain of the yeast Gcn4 protein (denoted as z_1) (Fig. 2, constructs IX and X; refs. 18–21). (The reason for linking N_{ub} to z_1 will

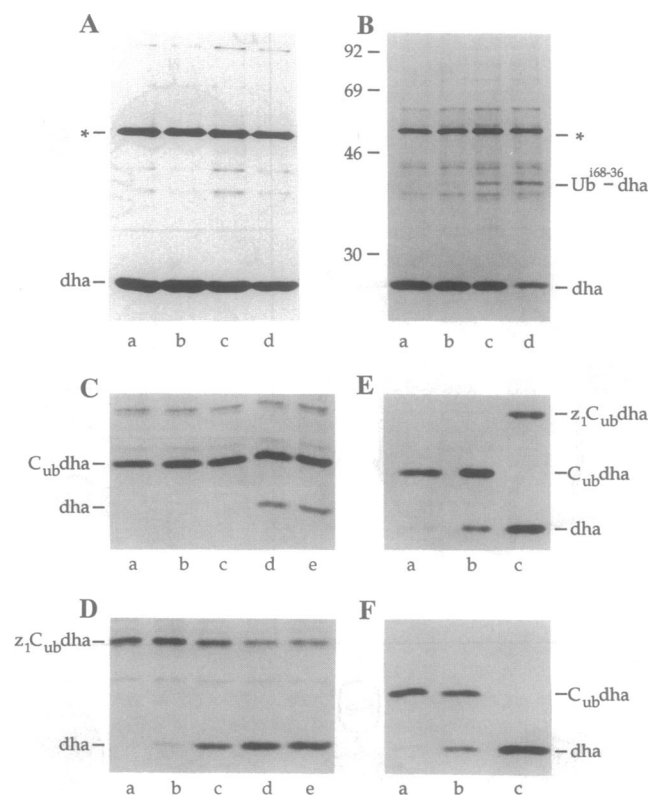


FIG. 3. Kinetic and equilibrium aspects of ubiquitin reconstitution *in vivo*. (A) Lane a, *S. cerevisiae* cells expressing Ub-dha (Fig. 2, construct I) were labeled for 5 min with [35 S]methionine, followed by extraction of proteins, immunoprecipitation with anti-ha antibody, and SDS/PAGE. Lanes b–d, same as lane a but with the Ub moiety containing, respectively, Val, Ala, or Gly instead of wild-type Ile at position 13 (Fig. 2, constructs II–IV). (B) Lane a, same as lane a in A but with a labeling time of 2 min and with *S. cerevisiae* expressing Ubⁱ⁶⁸-dha (Fig. 2, construct V). Lanes b–d, same as lanes b–d in A but with the single-residue replacements at position 13 in the Ubⁱ⁶⁸ moiety (constructs VI–VIII). (C) Lane a, same as lane a in B but with *S. cerevisiae* expressing C_{ub} -dha (construct IX). Lane b, C_{ub} -dha was coexpressed with N_{ub}^{G13} - z_1 (construct XIII). Lane c, C_{ub} -dha was coexpressed with N_{ub}^{A13} - z_1 (construct XII). Lane d, C_{ub} -dha was coexpressed with N_{ub}^{V13} - z_1 (construct XI). Lane e, C_{ub} -dha was coexpressed with N_{ub}^{WT} - z_1 (construct X). (D) Same as lane a in C but with *S. cerevisiae* expressing z_1C_{ub} -dha (construct XIV). Lane b, z_1C_{ub} -dha was coexpressed with N_{ub}^{G13} - z_1 . Lane c, z_1C_{ub} -dha was coexpressed with N_{ub}^{A13} - z_1 . Lane d, z_1C_{ub} -dha was coexpressed with N_{ub}^{V13} - z_1 . Lane e, z_1C_{ub} -dha was coexpressed with N_{ub}^{WT} - z_1 . (E) Whole-cell extracts of *S. cerevisiae* expressing C_{ub} -dha (lane a), or C_{ub} -dha and N_{ub}^{G13} - z_1 (lane b), or z_1C_{ub} -dha and N_{ub}^{G13} - z_1 (lane c) were fractionated by SDS/PAGE and analyzed by immunoblotting with anti-ha antibody. (F) Same as E but with *S. cerevisiae* expressing C_{ub} -dha (lane a), or C_{ub} -dha and N_{ub}^{A13} - z_1 (lane b), or z_1C_{ub} -dha and N_{ub}^{A13} - z_1 (lane c). Ubⁱ⁶⁸-dha, free reporter (dha), C_{ub} -dha, and z_1C_{ub} -dha are indicated. The asterisk in B denotes an unrelated *S. cerevisiae* protein that crossreacted with anti-ha antibody (11, 14).

become clear later.) When expressed by itself, C_{ub} -dha remained largely uncleaved (Fig. 4A, lanes a–c). However, coexpression of C_{ub} -dha and N_{ub}^{WT} - z_1 resulted in the cleavage of C_{ub} -dha, yielding dha, which accumulated during a 30-min chase (Fig. 4A, lanes d–f). This cleavage was slow in comparison to the cleavage of a fusion containing wild-type Ub (Fig. 3A, lane a). We conclude that the cleavage of C_{ub} -dha requires the presence of N_{ub} and is the consequence of an *in vivo* association between the C_{ub} and N_{ub} fragments. This association results in at least transient formation of a Ub moiety which is similar enough to native Ub to be a substrate of UBPs. That fragments of a protein can associate to form a functional, quasi-native species has been demonstrated

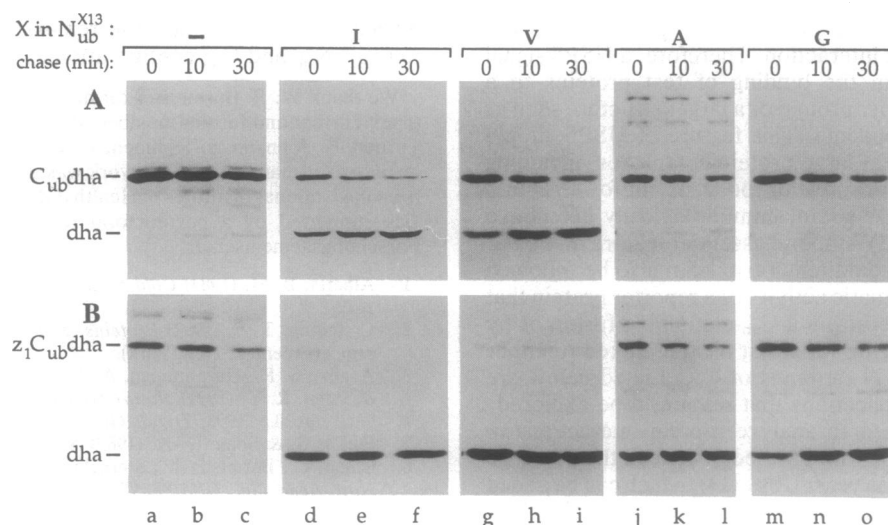


FIG. 4. Reconstitution of the folded ubiquitin from its coexpressed fragments: enhancement by cis-linked interacting polypeptides. (A) Lanes a–c, *S. cerevisiae* cells expressing C_{ub}-dha (Fig. 2, construct IX) were subjected to incubation for 5 min with [³⁵S]methionine, followed by a chase for 0, 10, and 30 min, extraction of proteins, immunoprecipitation with anti-ha antibody, and SDS/PAGE. Note a slow degradation of C_{ub}-dha and the absence of the cleavage that yields dha. Lanes d–f, same as lanes a–c, but C_{ub}-dha was coexpressed with N_{ub}^{Wt}-Z₁ (construct X). Lanes g–i, C_{ub}-dha was coexpressed with N_{ub}^{V13}-Z₁ (construct XI), which bore Val instead of wild-type Ile at position 13 of Ub. Lanes j–l, C_{ub}-dha was coexpressed with N_{ub}^{A13}-Z₁ (construct XII). Lanes m–o, C_{ub}-dha was coexpressed with N_{ub}^{G13}-Z₁ (construct XIII). (B) Lanes a–c, same as lanes a–c in A but with z₁C_{ub}-dha (construct XIV) instead of C_{ub}-dha. Lanes d–f, z₁C_{ub}-dha was coexpressed with N_{ub}^{Wt}-Z₁. Lanes g–i, z₁C_{ub}-dha was coexpressed with N_{ub}^{V13}-Z₁. Lanes j–l, z₁C_{ub}-dha was coexpressed with N_{ub}^{A13}-Z₁. Lanes m–o, z₁C_{ub}-dha was coexpressed with N_{ub}^{G13}-Z₁.

both *in vitro* and *in vivo* for a variety of proteins other than Ub (35–41).

The efficiency of Ub reconstitution depended on the conformational stability of its N-terminal subdomain: coexpression of C_{ub}-dha with either N_{ub}^{G13}-Z₁ or N_{ub}^{A13}-Z₁ (Fig. 2, constructs IX, XII, and XIII), bearing Gly or Ala instead of Ile at position 13, resulted in virtually no cleavage of C_{ub}-dha, in contrast to the results with either N_{ub}^{Wt}-Z₁ or N_{ub}^{V13}-Z₁ (Fig. 2, constructs X and XI), which bore either wild-type Ile or Val, a hydrophobic residue larger than Ala and Gly, at position 13 (Fig. 1 c and d and Fig. 4A, lanes j–o; compare with lanes d–i). These results, together with the discovery that the UBP-mediated cleavage of a Ub fusion requires folded Ub (11), led to development of an assay for *in vivo* protein interactions, as shown below.

Split Ubiquitin as a Proximity Sensor. We asked whether the linking of two polypeptides that interact *in vivo* to N_{ub} and C_{ub} facilitates reconstitution of Ub. C_{ub}-dha was linked to a region of *S. cerevisiae* Gcn4 (residues 235–281) that contained the leucine zipper homodimerization domain (denoted as z₁) (18–21). In the resulting z₁C_{ub}-dha, a 32-residue linker was inserted between z₁ and C_{ub} (Fig. 2, construct XIV) to ensure that N_{ub} and C_{ub} subdomains could be spatially proximal within a z₁-mediated complex between z₁C_{ub}-dha and N_{ub}-Z₁. When expressed by itself, z₁C_{ub}-dha remained uncleaved and was slowly degraded during the chase (Fig. 4B, lanes a–c). However, coexpression of z₁C_{ub}-dha and N_{ub}^{G13}-Z₁, bearing Gly instead of Ile at position 13 of Ub, resulted in a significant cleavage of z₁C_{ub}-dha (yielding dha) in the course of a 30-min chase (Fig. 2, constructs XIII and XIV; Fig. 4B, lanes m–o). By contrast, no such cleavage was observed when N_{ub}^{G13}-Z₁ was coexpressed with C_{ub}-dha, which lacked z₁ (Fig. 4A, lanes m–o). Similar results (but with faster cleavage of z₁C_{ub}-dha) were obtained upon coexpression of z₁C_{ub}-dha and N_{ub}^{A13}-Z₁ (Fig. 4, lanes j–l). Moreover, the enhancement of Ub reconstitution by z₁-z₁ interactions was observed even with pairs of Ub fragments that could yield Ub by themselves (in the absence of linked z₁). Specifically, whereas the coexpression of C_{ub}-dha and N_{ub}^{Wt}-Z₁ or N_{ub}^{V13}-Z₁ resulted in detectable but slow cleavage of C_{ub}-dha that was still incomplete after 30 min of chase, coexpression of z₁C_{ub}-

dha and N_{ub}^{Wt}-Z₁ or N_{ub}^{V13}-Z₁ resulted in the nearly complete cleavage of z₁C_{ub}-dha (yielding dha) by the end of a 5-min pulse (Fig. 4, lanes d–i). The temporal resolution of this assay could be increased by shortening the labeling time from 5 to 2 min. For example, the fraction of z₁C_{ub}-dha cleaved by the end of a 2-min pulse progressively increased when z₁C_{ub}-dha was coexpressed with N_{ub}^{G13}-Z₁, N_{ub}^{A13}-Z₁, N_{ub}^{V13}-Z₁, or N_{ub}^{Wt}-Z₁ (Fig. 3D, lanes b–e). By contrast, no cleavage of z₁C_{ub}-dha was observed when it was expressed by itself (Fig. 3D, lane a), or when N_{ub}^{A13}-Z₁ or N_{ub}^{G13}-Z₁ were coexpressed with C_{ub}-dha, which lacked the z₁ zipper (Fig. 3C, lanes b and c).

To determine steady-state levels of test proteins, cell extracts were analyzed by immunoblotting with anti-ha antibody (Fig. 3 E and F). When C_{ub}-dha was expressed by itself, it remained largely uncleaved (Fig. 3 E and F, lanes a). When N_{ub}^{A13}-Z₁ was coexpressed with C_{ub}-dha, a fraction of C_{ub}-dha was cleaved to yield dha (Fig. 3F, lane b). However, when N_{ub}^{A13}-Z₁ was coexpressed with z₁C_{ub}-dha, virtually all of z₁C_{ub}-dha was cleaved to yield dha (Fig. 3F, lane c). Similar results were obtained with N_{ub}^{G13}-Z₁, except that a significant fraction of z₁C_{ub}-dha remained uncleaved in the presence of N_{ub}^{G13}-Z₁ (Fig. 3E, lanes b and c).

DISCUSSION

Selecting appropriate Ub fragments, altering one of them to reduce the rate of Ub reconstitution by fragments alone, and linking these fragments to a pair of test polypeptides yielded USPS, an assay for kinetic and equilibrium aspects of *in vivo* protein interactions. Features of USPS that distinguish it from the two-hybrid technique (5–8) include the possibility of monitoring a protein–protein interaction as a function of time, at the natural sites of this interaction in a living cell. Enhancement of Ub reconstitution by interacting polypeptides linked to fragments of Ub stems from a local increase in concentration of one Ub fragment in the vicinity of the other. This increase results in a higher probability of the two Ub fragments associating to form a quasi-native Ub moiety, an event detected through the irreversible cleavage of the fusion by UBPs (Fig. 1e).

USPS detects a spatial proximity of proteins but not necessarily their direct interaction. Therefore, a USPS signal might also result from the binding of test proteins to a common ligand—another protein or a larger structure such as a microtubule or a ribosome. This feature of USPS should allow *in vivo* analyses of large protein complexes, including those that may be too unstable or too transient for detection by other methods. A separate, immunologically detectable reporter domain (Fig. 1) is not an essential feature of USPS: the cleavage of a C_{ub} -containing fusion can also be followed by measuring the enzymatic activity of a reporter protein that is inactive until released from a C_{ub} -containing fusion or by using an antibody specific for a test protein linked to either the C terminus or the N terminus of C_{ub} . Listed below are some of the USPS applications that remain to be explored.

(i) USPS can be used to analyze protein interactions *in vitro*—in cell extracts or with purified fusions in the presence of a purified UBP such as yeast Ubp1 (13), which can be added at a desired time after the mixing of interacting species. The kinetics of Ub reconstitution from its fragments *in vitro* can be compared with analogous reconstitutions in living cells to address the influences of translation, chaperonins, macromolecular crowding, and other aspects of the *in vivo* condition. *In vitro* USPS can also be used to screen for peptide or nonpeptide ligands that interact with a ligand of interest.

(ii) Many proteins have more than one protein ligand. A version of USPS for a “many-body” interaction could be as follows. N_{ub} - P_1 , a fusion between N_{ub} and a protein of interest, P_1 , is coexpressed with two other proteins: P_2 - C_{ub} - R_1 , a fusion of C_{ub} , P_2 (a putative ligand of P_1), and a reporter R_1 , and a similarly designed P_3 - C_{ub} - R_2 fusion containing a different reporter, R_2 , and P_3 , another putative ligand of P_1 . Comparing the kinetics of *in vivo* cleavage of P_2 - C_{ub} - R_1 and P_3 - C_{ub} - R_2 in the presence of N_{ub} - P_1 should provide information about relative affinities of P_1 for P_2 and P_3 and about kinetic aspects of these interactions as well.

(iii) Lateral diffusion and interactions of membrane proteins underlie membrane-based reactions such as activation of receptors by hormones and adhesion between cells. In this version of USPS, the two Ub subdomains can be positioned within cytosol-exposed regions of two membrane proteins.

(iv) A USPS-based screen for genes whose products interact with a protein of interest P_1 can employ a reporter R whose function is incompatible with an N-terminal extension such as P_1 - C_{ub} . The reporter would be activated upon Ub reconstitution (Fig. 1e) and the cleavage of P_1 - C_{ub} - R that yields R . In this approach, cells expressing P_1 - C_{ub} - R are transformed with an expression library encoding random translational fusions to a mutant N_{ub} moiety and screened for cells expressing active reporter.

USPS was demonstrated here with homodimerizing polypeptides of the leucine zipper type (Figs. 2 and 4). In addition, we recently used USPS to detect and analyze *in vivo* interactions between *S. cerevisiae* Sec62 protein and the signal sequences of either the *SUC2*-encoded invertase or the *MFal*-encoded precursor of α -factor, a mating pheromone. Sec62 is an integral membrane protein and essential component of the translocation complex which mediates the transport of proteins bearing signal sequences across the endoplasmic reticulum membrane (42). The USPS assay detected specific, transient interactions between Sec62 and the signal sequences; it also made possible a kinetic analysis of these *in vivo* interactions, which have previously been demonstrated using photocrosslinking in a cell-free system (43). The transient proximity between a signal sequence and the membrane-embedded Sec62 could be detected by using several N_{ub} and C_{ub} arrangements in Sec62 and signal sequence-bearing test proteins. Together with the results of the present work, these findings (unpublished data) illustrate the versatility of USPS, its sensitivity to relatively weak and transient

protein interactions, and the remarkable flexibility of “allowed” N_{ub} and C_{ub} configurations within test proteins.

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